

Video Article

Imaging InlC Secretion to Investigate Cellular Infection by the Bacterial Pathogen *Listeria monocytogenes*

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Abstract

Bacterial intracellular pathogens can be conceived as molecular tools to dissect cellular signaling cascades due to their capacity to exquisitely manipulate and subvert cell functions which are required for the infection of host target tissues. Among these bacterial pathogens, *Listeria monocytogenes* is a Gram positive microorganism that has been used as a paradigm for intracellular parasitism in the characterization of cellular immune responses, and which has played instrumental roles in the discovery of molecular pathways controlling cytoskeletal and membrane trafficking dynamics. In this article, we describe a robust microscopical assay for the detection of late cellular infection stages of *L. monocytogenes* based on the fluorescent labeling of InlC, a secreted bacterial protein which accumulates in the cytoplasm of infected cells; this assay can be coupled to automated high-throughput small interfering RNA screens in order to characterize cellular signaling pathways involved in the up- or down-regulation of infection.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51043/>

Introduction

The Gram positive bacterium *Listeria monocytogenes* is a food-borne pathogen that invades host cells, disrupts its internalization vacuole and replicates in the cytoplasm of host cells¹. *L. monocytogenes* ease of manipulation in the laboratory context (rapid growth, low toxicity for healthy individuals) associated with the persistence of bacterial virulence traits observed in cellular and animal models (hemolytic activity, leukocytosis) allowed its initial use in the 1960s as a major model for the study of intracellular parasitism and for the establishment of the theoretical foundations of cellular immunity against infection². In the late 1980s and early 1990s, the dissection of the bacterial intracellular cycle³ as well as the molecular characterization of the most important bacterial virulence factors⁴⁻⁷ favored the use of *L. monocytogenes* as a key molecular tool for the manipulation and study of host cell functions. The presence of avirulent (*L. innocua*) and virulent (*L. monocytogenes*) species in the *Listeria* genus paved the way for comparative genomic studies⁸ that, together with the recent establishment of the complete *L. monocytogenes* transcriptome⁹, have increased our understanding of the evolution of *L. monocytogenes* as a human pathogen and as a model system for infection studies¹⁰.

L. monocytogenes induces its internalization into host cells upon interaction of the bacterial surface proteins InlA and InlB with their host cell receptors E-cadherin and Met, respectively¹¹⁻¹². Initial candidate-based studies led to the identification of the α/β catenins-actin link as a significant component of the InlA-invasion pathway¹³ and of the phosphoinositide 3-kinase (PI 3-K) as a critical effector of the InlB-dependent invasion cascade¹⁴⁻¹⁵. Proteomic and functional-based assays subsequently allowed the identification of novel cytoskeletal elements¹⁶ and lipid second messengers¹⁷ required for host cell invasion. Transcriptional studies¹⁸ and mass spectrometry-based quantitative proteomics¹⁹ have recently shed new light concerning the activation of host signaling cascades and the repression of immune responses during *L. monocytogenes* infection. Systems biology approaches based on the inactivation of large sets of genes (kinomes, complete genomes) by small interfering RNA (siRNA) silencing have recently opened new avenues for the analysis of global host signaling cascades in the context of specific cellular functions, including phagocytosis and pathogen internalization²⁰. Genome-wide siRNA screens have been previously performed to investigate cellular cascades required for infection of *L. monocytogenes* in phagocytic *Drosophila* S2 cells²¹⁻²², but this type of analysis has not been performed in non-phagocytic cells, which represent critical targets for infection *in vivo*.

We have optimized a protocol for the microscopical detection of late stages of infection by *L. monocytogenes* that is suited for high-throughput siRNA studies of bacterial entry within epithelial cells. Our assay takes advantage of a highly invasive *L. monocytogenes* strain that presents

a point mutation in PrfA, the major transcriptional regulator of *L. monocytogenes* virulence factors⁶: this mutation (named PrfA*) renders PrfA constitutively active²³ and leads to an increased expression of the invasion proteins InlA and InlB, therefore favoring bacterial entry in otherwise poorly infected non-phagocytic cells. Our readout for infection is based on the detection of the cytosolic accumulation of the secreted bacterial protein InlC: this molecule is a pleiotropic effector that is expressed preferentially by intra-cytoplasmic *L. monocytogenes*⁹ and which participates not only in the bacterial cell-to-cell spread²⁴ but which also modulates host immune responses²⁵. The fluorescent labeling of InlC secretion by intracellular bacteria not only allows to clearly distinguish infected from non-infected cells, but also represents an end-point readout that can be used to subsequently dissect infection in its different steps: entry, vacuolar escape, cytosolic bacterial proliferation and cell-to-cell spread. This microscopy-based protocol can be coupled to siRNA screens therefore to study cellular pathways involved in the infection of host cells by *L. monocytogenes*.

Protocol

1. Preparation of Cellular and Bacterial Cultures, Transfection Tools and Primary Antibodies

1. Prepare a fresh agar plate to isolate individual *L. monocytogenes* colonies from a bacterial glycerol stock (50% glycerol/50% saturated bacterial liquid overnight culture) kept at -80 °C.
 1. Using an aluminum rack (kept at -80 °C) to transport a frozen bacterial glycerol stock, streak bacteria on a Brain Heart Infusion (BHI) agar plate.
 2. Incubate the plate at 37 °C during 48 hr (or until individual bacterial colonies can be isolated).
 3. Keep this working plate afterwards at 4 °C during a maximum time of 1 month (it will be used to seed liquid cultures).
 4. In our protocol, we use the *L. monocytogenes* serotype 1/2a EGDe.PrfA* strain, but as illustrated in **Figure 1**, the *L. monocytogenes* serotype 4b P14.PrfA* strain gives similar results.
2. HeLa ATCC CCL2 cells are grown in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in the absence of antibiotics.
3. Independent pools of scrambled (control) and anti-Met siRNAs are loaded in black 384-well microscopy cell culture plates.
 1. Dilute 160 pmol of siRNA in 500 µl of RNase-free water and add 5 µl of this solution per well (final concentration: 1.6 pmol).
 2. Keep this plate at -20 °C for a maximum period of 1 year.
4. Polyclonal rabbit antibodies against the bacterial protein InlC have been obtained by immunization using an InlC-GST recombinant protein as described previously²⁵.

2. Reverse siRNA Cell Transfection

1. 72 hr before infection bring to room temperature a black 384-well microscopy cell culture plate containing 1.6 pmol siRNA in 5 µl of RNase-free water in each well.
2. Centrifuge the plate for 3 min at 300 rcf at room temperature to bring down siRNA which could have been deposited in the walls of the wells.
3. Prepare room temperature DMEM supplemented with 0.4% Lipofectamine RNAiMAX.
4. Add 25 µl of the DMEM/RNAiMAX transfection medium to each well (do not incubate the transfection medium longer than 20 min before adding it to the siRNA).
5. Move the plate back and forth to mix the siRNA with the transfection solution, then keep the plate for 1 hr at room temperature to allow siRNA-Lipofectamine complexes to form.
6. Wash HeLa cells from a confluent- or sub-confluent cell culture flask once with 10 ml 37 °C pre-warmed PBS.
7. Detach HeLa cells by adding 1 ml 37 °C prewarmed trypsin to the cell culture flask and incubate for 3 to 5 min at 37 °C.
8. Re-suspend cells in 10 ml 37 °C prewarmed DMEM supplemented with 16% FBS.
9. Count cells and prepare a cell suspension of 12,000 cells per ml in DMEM supplemented with 16% FBS.
10. Add 50 µl of the cell suspension to each well in the 384-well plate.
11. Move the plate quickly back and forth to distribute cells and let cells settle down for 10 min at room temperature.
12. Seal the plate with Parafilm and keep it for 72 hr in a humidified 5% CO₂-containing atmosphere at 37 °C.

3. Cellular Infection and Staining

1. The day before infection, take a single colony of *L. monocytogenes* from the BHI agar plate and resuspend it in 5 ml of liquid BHI medium in a 15 ml polystyrene tube.
2. Incubate overnight at 37 °C in a shaking device to allow for bacterial growth.
3. The day of the infection, wash 1 ml of the overnight *L. monocytogenes* culture by centrifuging 2 min at 10,600 rcf on a table-top centrifuge.
4. Discard the supernatant (which contains the secreted cytotoxin listeriolysin O) and re-suspend the pellet in 1 ml of PBS (repeat the washing step 3 more times).
5. Read the bacterial optical density at 600 nm and estimate the number of bacteria (OD=1 is equivalent to 1E9 bacteria/ml).
6. Prepare the adequate *L. monocytogenes* dilution in DMEM supplemented with 1% FBS: using highly invasive strains like EGDe.PrfA*, we suggest the use of 5E4 bacteria in 30 µl of medium per well (the multiplicity of infection is estimated as 25 for 2,000 cells).
7. Remove the cell culture medium in each well (80 µl) and replace it by adding 30 µl of the *L. monocytogenes*-containing medium.
8. Centrifuge the plate at 200 rcf for 5 min at room temperature to synchronize the infection process.
9. Incubate the plate for 1 hr in a humidified 5% CO₂-containing atmosphere at 37 °C on a prewarmed aluminum block.
10. Remove the *L. monocytogenes*-containing medium from each well and add 30 µl of prewarmed DMEM supplemented with 10% FBS and 40 µg/ml gentamicin to kill extracellular *L. monocytogenes*.

11. Incubate for 4 hr in a 5% CO₂-containing atmosphere at 37 °C on a prewarmed metal block.
12. Prepare a solution of PBS supplemented with 8% formaldehyde (this solution should be prepared fresh so that the formaldehyde monomers, rather than the paraformaldehyde polymers, are used).
13. Without discarding the cell culture medium, add 30 µl of PBS supplemented with 8% formaldehyde (final concentration: 4%) and incubate for 15 min at room temperature.
14. Remove the fixative and wash the cells three times with 80 µl of PBS per well (keep the cells in a final volume of 80 µl of PBS per well).
15. Prepare a 1:250 dilution of the rabbit anti-InIC serum in PBS supplemented with 0.2% saponin.
16. Add 10 µl of the primary antibody solution to each well after removing the PBS and incubate for 30 min at room temperature.
17. Discard the primary antibody solution and wash four times with 40 µl of PBS per well.
18. Dilute the secondary Alexa Fluor 546-coupled anti-rabbit antibody (1:250), the DAPI solution (1:1,500) and phalloidin-Dy647 (1:150) in PBS supplemented with 0.2% saponin.
19. Add 10 µl of this secondary staining solution to each well and incubate for 30 min at room temperature.
20. Discard the secondary staining solution and wash four times with 40 µl of PBS per well (leave a final volume of 40 µl in each well and seal the plate).
21. The plate can be imaged immediately or can be stored at 4 °C protected from light (cover with aluminum foil) for subsequent analysis.

4. Image Acquisition and Analysis

1. Acquire images in three different channels (350 nm, 546 nm and 647 nm) using a 10X objective mounted on an automated microscope (acquire preferentially 9 images per well).
2. The InIC signal can be measured using image analysis software like CellProfiler that allows automated segmentation of nuclei and cell bodies using the DAPI and the phalloidin staining, respectively.

Representative Results

Fluorescent labeling of cytoplasmic InIC provides a robust readout for cell infection by *L. monocytogenes*, as illustrated in **Figure 1**: the central cell in the micrograph is highly infected by the strain P14.PrfA²³ as it can be observed in the phase contrast image (arrowheads, **Figure 1A**) and it is confirmed by the DAPI signal where individual bacteria can be clearly distinguished (**Figure 1B**). The InIC staining (superposed to the DAPI staining in **Figure 1C**) depicts how this secreted protein densely accumulates in the cytosol of infected cells and allows an unambiguous detection of the infected host cell morphology. Staining of the actin cytoskeleton with fluorescent phalloidin (**Figure 1D**) provides information concerning the morphology of the complete inoculated cellular monolayer and further illustrates how neighboring non-infected cells (cell nuclei marked with an asterisk) do not display any InIC labeling. It is worth to mention that, since InIC cytosolic levels are dependent on the number of cytoplasmic bacteria, we have noted a correlation between the InIC signal detected by immunofluorescence and the number of *L. monocytogenes* per cell: as observed in **Figure 1**, neighboring cells infected with low bacterial numbers display a reduced InIC staining which can be quantified. Interestingly, it is also possible to observe that InIC is easily detected in the protrusions formed by bacteria caught in the process of cell-to-cell spread (**Figure 1C**) as observed also with the actin staining (**Figure 1D**). Of note, bacteria are not directly stained in our protocol but since the 488 nm channel is not used, *L. monocytogenes* can be labeled using a specific anti-bacterial serum; otherwise, GFP-expressing bacteria can alternatively be used.

Using imaging analysis tools such as the public software CellProfiler (Broad Institute, www.cellprofiler.org), it is possible to segment cells and to measure the InIC signal in individual infected cells, which can be used to estimate an infection index for a specific experimental condition. As shown in **Figure 2**, the labeling of the nuclei with DAPI (**Figure 2A**) and the actin cytoskeleton with phalloidin (**Figure 2C**) provide the information required for cellular segmentation: the nuclei are used as reference objects for the identification of individual cells (**Figure 2B**) and the cytoplasm is subsequently identified using a spread function from the nuclei that takes into account the cytoskeletal signal (**Figure 2D**). Finally, the intensity of the InIC signal can be quantified for each identified cellular object (**Figures 2E and 2F**) to estimate the number of infected cells by setting a threshold for the InIC intensity that we consider as negative. The infection index is calculated as the number of infected cells divided by the total number of cells in the population.

Our protocol can be coupled to siRNA screens to investigate the function of large panels of target molecules in the infection of host cells by *L. monocytogenes*. Controls are required to validate the efficiency of transfection: for example, siRNA targeting Kif11 is a commonly used control for transfection that leads to cell death, and therefore the absence of cells at the end of the assay is an indication that transfection proceeded efficiently. To specifically address the *L. monocytogenes* invasion process, siRNA inactivation of the cellular receptor Met in HeLa cells leads to a major inhibition of bacterial entry into host cells and leads to very low levels of InIC-positive cells in an inoculated cellular monolayer (**Figure 3A**) as compared to cells treated with a scrambled siRNA control (**Figure 3B**). The function of candidate unknown molecules can be investigated with our assay using this known cell molecule as standard.

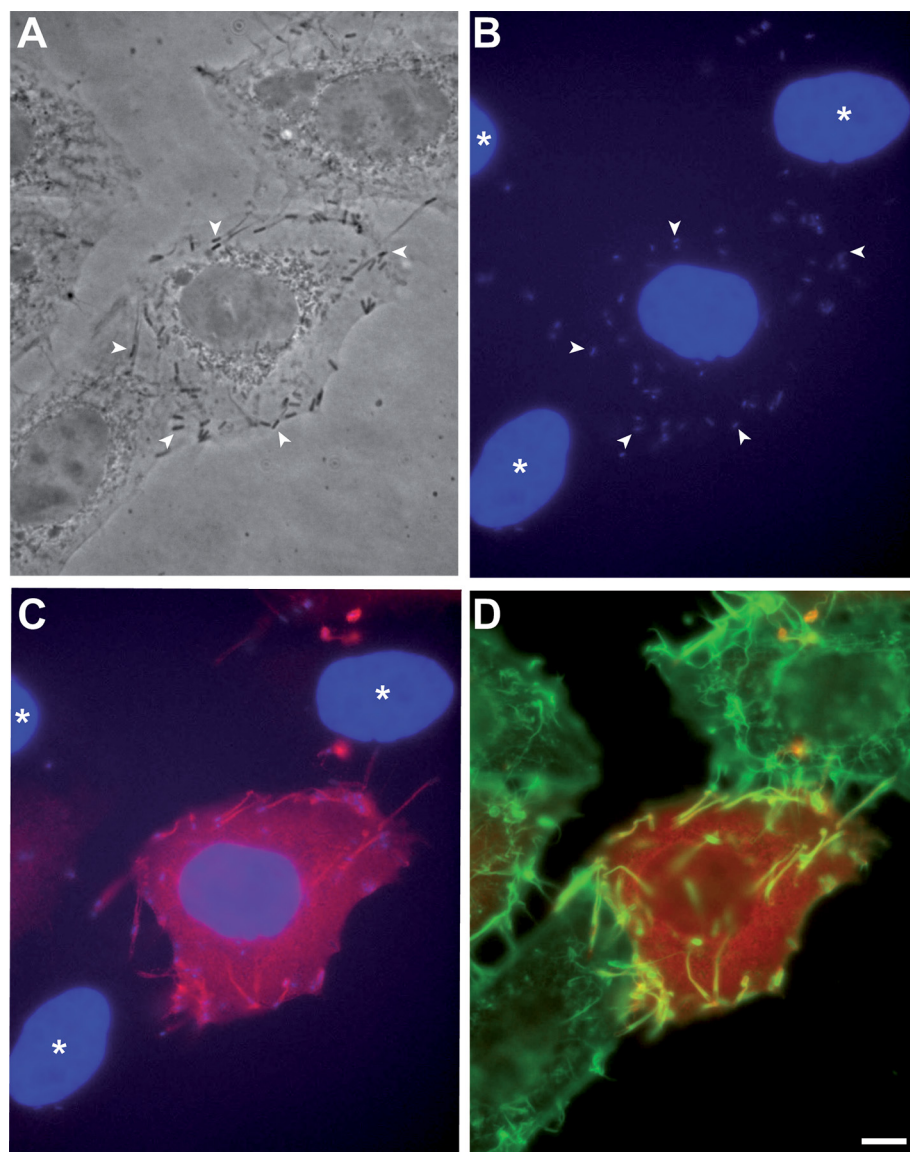


Figure 1. Detection of InIC labeling in HeLa cells infected with *L. monocytogenes* strain P14.PrfA*. HeLa CCL2 cells have been infected as described in our protocol, processed for immunofluorescence and imaged using a 63X objective. **(A)** Phase contrast image, several P14.PrfA* bacteria are indicated by arrowheads. **(B)** DAPI staining (blue), the same individual bacteria as in **(A)** are labeled by arrowheads. **(C)** Superposition of the DAPI staining (blue) and the InIC staining (red), the nuclei of uninfected cells are labeled by an asterisk. **(D)** Superposition of the InIC (red) and actin (green) signals. Bar: 5 μ m. [Click here to view larger figure.](#)

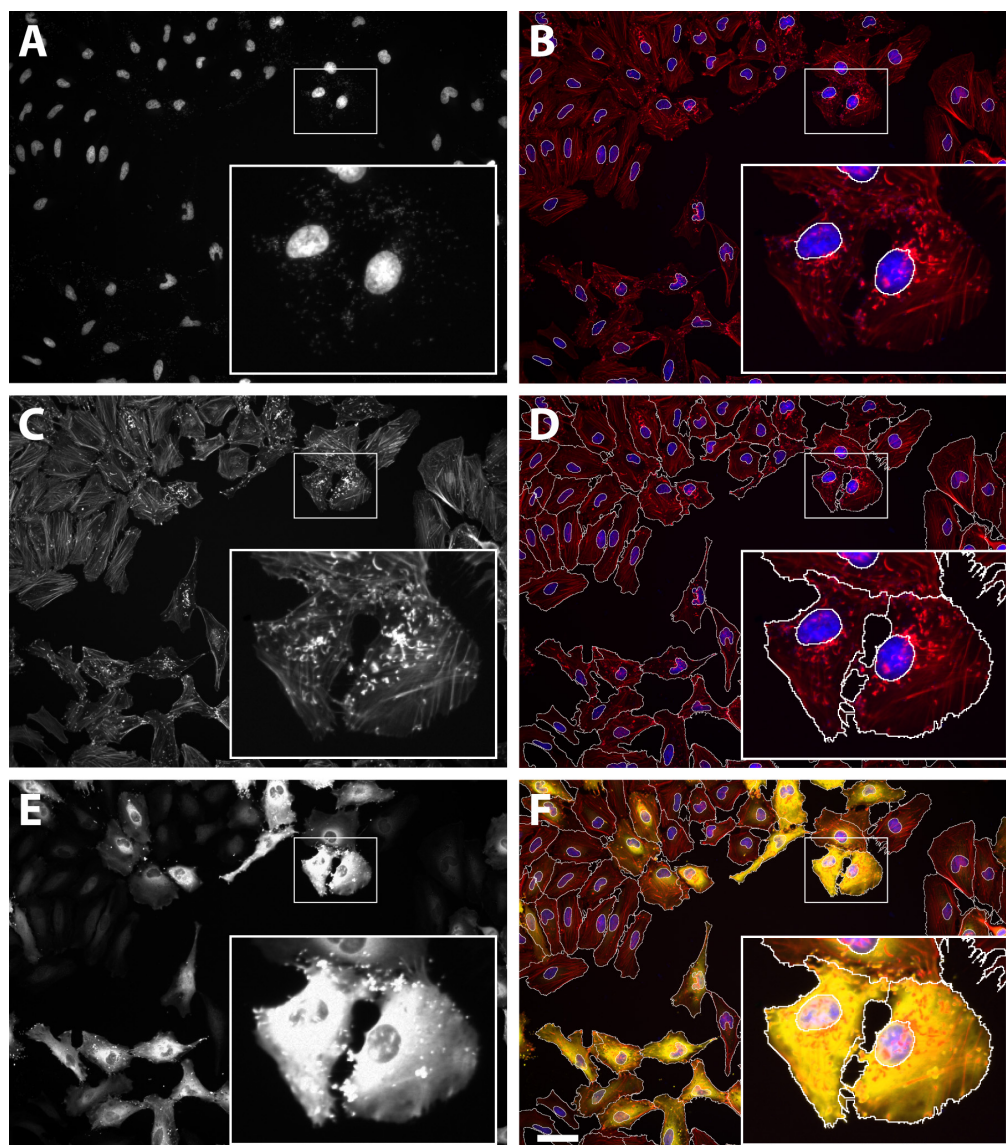


Figure 2. Segmentation analysis of HeLa cells infected with *L. monocytogenes* strain EGDe.PrfA*. HeLa CCL2 cells were infected as described in our protocol, processed for immunofluorescence and imaged using a 10X objective. (A) DAPI signal. (B) Superposition of the DAPI signal (blue) displayed in (A) and actin signal (red) displayed in (C) showing the segmentation of the nuclei (view enlarged inset). (C) Actin signal. (D) Same image as (B) showing the segmentation of the cells. (E) InIC signal. (F) Same image as (D) with superposition of the InIC staining (yellow). Bar: 50 μ m. [Click here to view larger figure.](#)

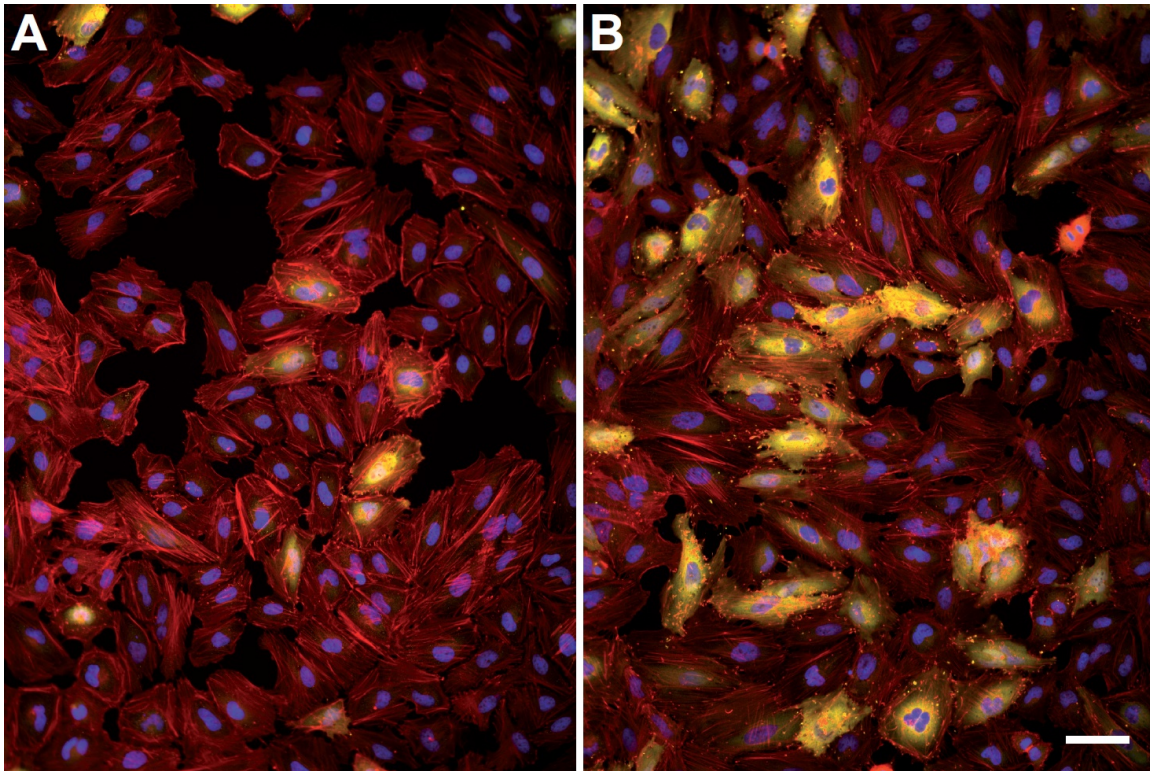


Figure 3. Variation of the InIC labeling between cells inactivated for Met and control cells. HeLa CCL2 cells were initially reverse-transfected with a pool of four siRNAs targeting the host cell receptor Met; 72 hr after transfection, cells were infected as described, processed for immunofluorescence and imaged using a 10x objective. (A) Superposition of the DAPI (blue), actin (red) and InIC (yellow) in cells inactivated for Met. (B) Same channels as in (A) concerning cells treated with a scrambled siRNA control. Bar: 50 μ m. [Click here to view larger figure.](#)

Discussion

Several parameters are critical for the success of our InIC-detection protocol, including the use of healthy cell lines displaying a sufficiently large cytoplasm to allow an unambiguous detection of the InIC signal. In the assay we present in this article we propose the use of HeLa CCL2 cells, which are particularly well suited for our assay due to the extension of their cytosolic space; other HeLa clones such as HeLa Kyoto cells display a smaller cytoplasm but can be used with our infection protocol (HeLa Kyoto cells are particularly well adapted for segmentation analysis since cells do not overlap with neighboring cells, which is often the case for HeLa CCL2 cells). Cells presenting a very small cytoplasm such as polymorphonuclear cells or macrophages are not recommended to use in combination with our protocol.

A limitation of an imaging protocol as the one we present in here is the fact that a minimal amount of cells should be infected, in a given monolayer in control conditions, in order to provide the system with enough resolutive power: otherwise, if few cells are infected, it becomes difficult to determine changes in infection rates, especially when investigating the function of potential molecular candidates that are expected to reduce infection. This limitation represents a real problem when using HeLa cells, which express Met as the only surface receptor for the *L. monocytogenes* protein InlB and are therefore poorly invaded by most *L. monocytogenes* strains. One alternative is to use a very high multiplicity of infection (MOI>100) which may increase the number of invasive bacteria but also increases the risk of cell damage due to higher levels in the cell culture medium of the bacterial pore forming toxin listeriolysin O (LLO), which displays a very potent cytotoxic activity. Other alternative is the use of super-invasive strains such as the EGDe.PrfA* strain presented in our protocol, which allows the use of a low MOI (<25) and reduces the amount of LLO-dependent cytotoxic effects; results obtained with a *L. monocytogenes* super-invasive strain can be subsequently validated using other less virulent bacterial strains in other types of assays (see below). A third alternative is to use a different cell line which expresses both E-cadherin and Met: it is the case for cell lines such as the trophoblast-like Jeg3 or BeWo cells, or the colon carcinoma LoVo cells, which can be invaded by both the InlA- and the InlB-dependent entry pathways; in these cell lines, higher rates of cell infection can be reached using *L. monocytogenes* strains such as EGDe, the parental strain of EGDe.PrfA*. It should be taken into account, however, that redundancy of functions in both pathways can lead to compensation effects in one pathway when an effector is inactivated in the other pathway, rendering the analysis of results difficult. It is also important to mention that cells should not be over-infected in order to provide the system with a good dynamic range allowing the detection of events that increase infection: in our particular protocol, our MOI leads to an optimal infection rate of 30%.

Alternative methods to study the invasion of host cells by *L. monocytogenes* include the classical gentamicin invasion assay²⁶ which is based on the killing of extracellular bacteria by the addition of gentamicin to the cell culture medium after an initial period of bacterial infection (similar to the procedure presented in the first part of our infection protocol) and invasion is scored by plating the surviving intracellular bacteria on agar plates and counting the number of colony forming units (CFUs) that grow on these plates. This method presents the advantage of using the most direct readout for infection, which is the precise number of invasive bacteria that are actually protected from the gentamicin treatment in the cytoplasmic space of host cells; however, this method presents a single readout for infection and once the host cells are lysed to release intracellular CFUs, there is no longer a record to obtain information concerning the actual status of cells at the end point of the experiment. Our

protocol is based on an indirect method, the detection of the secreted InlC protein, but as mentioned previously, the levels of cytoplasmic InlC correlate with the number of cytosolic *L. monocytogenes* (Figure 1). Moreover, the intrinsic nature of our microscopy assay allows to clearly establish the precise status of the cells at the end of the infection: we can therefore extract information concerning global cellular morphology, actin cytoskeleton distribution, cell cycle phase, etc. and perform high content analysis of the infection. One important feature that can be extracted from this type of analysis is the population context and its influence on infection: indeed, recent work from Pelkmans and coworkers²⁷ demonstrated that the particular population context of a given cell (for example, its position from the periphery within a group of cells in an islet) affects several cellular functions including endocytosis and susceptibility to virus infection. Our assay presents therefore the potential for the extraction of this information.

Our method presents an additional advantage: since InlC is a late readout of infection due to its secretion by cytoplasmic bacteria, host signaling pathways affecting the levels of InlC accumulation in host cells could potentially affect not only bacterial entry but also vacuolar escape, cytosolic proliferation and eventually cell-to cell spread. Hence, our method can be used as a primary readout for global infection and can be coupled to secondary assays to dissect the specific infection step that has been perturbed by the primary hit identified through siRNA screens.

Finally, it is important to mention that our method can be upscaled and fully automated using plate washer devices, increasing therefore the number of samples analyzed in a single experiment while decreasing the level of results variation when compared to experiments carried out manually.

Disclosures

We have nothing to disclose.

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